Post-print of: The Plant Cell, Vol. 24: 4621–4634, November 2012

www.plantcell.org/cgi/doi/10.1105/tpc.112.105403

**Cysteine-Generated Sulfide in the Cytosol Negatively Regulates Autophagy and Modulates the Transcriptional Profile in Arabidopsis[W]**

Consolación Álvarez, Irene García, Inmaculada Moreno, María Esther Pérez-Pérez, José L. Crespo, Luis C. Romero and Cecilia Gotor (1)

(1) Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, 41092 Seville, Spain

**Abstract**

In Arabidopsis thaliana, DES1 is the only identified l-Cysteine desulfhydrase located in the cytosol, and it is involved in the degradation of cysteine and the concomitant production of H2S in this cell compartment. Detailed characterization of the T-DNA insertion mutants des1-1 and des1-2 has provided insight into the role of sulfide metabolically generated in the cytosol as a signaling molecule. Mutations of L-CYS DESULFHYDRASE 1 (DES1) impede H2S generation in the Arabidopsis cytosol and strongly affect plant metabolism. Senescence-associated vacuoles are detected in mesophyll protoplasts of des1 mutants. Additionally, DES1 deficiency promotes the accumulation and lipidation of the ATG8 protein, which is associated with the process of autophagy. The transcriptional profile of the des1-1 mutant corresponds to its premature senescence and autophagy-induction phenotypes, and restoring H2S generation has been shown to eliminate the phenotypic defects of des1 mutants. Moreover, sulfide is able to reverse ATG8 accumulation and lipidation, even in wild-type plants when autophagy is induced by carbon starvation, suggesting a general effect of sulfide on autophagy regulation that is unrelated to sulfur or nitrogen limitation stress. Our results suggest that cysteine-generated sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile of Arabidopsis.

**INTRODUCTION**

Cys occupies a central position in plant primary and secondary metabolism. Beyond its importance as an amino acid, Cys is a precursor for a large number of essential biomolecules. Many plant defense compounds produced under biotic and abiotic stress derive from Cys (Rausch and Wachter, 2005) and contain sulfur moieties as functional groups. Recently, it has been suggested that Cys itself is an important determinant of the antioxidative capacity of the cytosol in Arabidopsis thaliana (López-Martín et al., 2008a, 2008b). The biosynthesis of Cys involves the sequential reaction of two enzymes, Ser acetyltransferase (EC 2.3.1.30), which synthesizes the intermediary product O-acetylserine from acetyl-CoA and Ser, and O-acetylserine(thiol)lyase (OASTL; EC 2.5.1.47), which incorporates the sulfide derived from the assimilatory reduction of sulfate with O-acetylserine to produce Cys. Plant cells contain different Ser acetyltransferase and OASTL enzymes in the cytosol, plastids, and mitochondria, resulting in a complex variety of isoforms and different subcellular Cys pools. In the model plant Arabidopsis, five Ser acetyltransferase (Howarth et al., 2003) and nine OASTL genes (Wirtz et al., 2004) have been identified.

In recent years, much progress has been made in understanding the most abundant OASTL enzymes in Arabidopsis, with a primary focus on their involvement in the primary sulfate assimilation pathway. We recently investigated DES1, a minor OASTL-like protein located in the cytosol, and showed that DES1 catalyzes the desulfuration of l-Cys to sulfide plus ammonia and pyruvate, rather than promoting Cys biosynthesis; thus, DES1 is a novel l-Cys desulfhydrase (EC 4.4.1.1) (Álvarez et al., 2010). A role of DES1 in plant metabolism was evidenced by the phenotypes of the T-DNA insertion mutants des1-1 and des1-2, which exhibit premature leaf senescence along with increased expression of senescence-associated genes and transcription factors. Disrupted DES1 function also significantly reduces the total Cys desulfuration activity in leaves, with a concomitant increase in the total Cys content (Álvarez et al., 2010). In response to plant pathogens, des1 mutants were found to behave as constitutive SAR mutants, exhibiting high resistance to biotrophic and necrotrophic pathogens, salicylic acid (SA) accumulation, and WRKY DOMAIN CONTAINING TRANSCRIPTION FACTOR54 and PATHOGENESIS RELATED1 induction (Álvarez et al., 2012). Taken together, these data highlight the importance of DES1 in the signaling and regulation of plant responses to various processes.

Hydrogen sulfide has increasingly been recognized as an important signaling molecule, of comparable importance to NO and CO in mammalian systems. The list of biological roles of H2S in various systems of the human body has rapidly expanded (Łowicka and Beltowski, 2007; Gadalla and Snyder, 2010; Kabil and Banerjee, 2010). Hydrogen sulfide is weakly acidic and dissociates in aqueous solution. Under physiological pH conditions (pH 7.4), one-third of the H2S present is undissociated, and the remaining two-thirds dissociate into H+ and HS−. HS− may subsequently dissociate into H+ and S2−, but this dissociation requires high pH conditions. Similar to NO and CO, H2S is lipophilic and permeates plasma membranes freely, although the ionized form HS− cannot permeate membranes (Kabil and Banerjee, 2010). H2S is endogenously produced in mammalian tissues by enzymatic reactions of l-Cys, primarily via two cytoplasmic enzymes, cystathionine-γ-lyase (EC 4.4.1.1) and cystathionine-β-synthetase (EC 4.2.1.22), which both use pyridoxal-5′-phosphate as a cofactor, with ammonia and pyruvate as by-products (Gadalla and Snyder, 2010). To our knowledge, DES1 is the only l-Cys desulfhydrase present in the Arabidopsis cytosol that catalyzes the desulfuration of l-Cys to sulfide plus ammonia and pyruvate and requires pyridoxal-5′-phosphate as a cofactor (Álvarez et al., 2010). DES1 may therefore be responsible for the release of metabolically regulated sulfide in this cellular compartment. The l-Cys desulfurases (EC 2.8.1.7) also catalyze Cys desulfuration required for iron-sulfur cluster, thiamine, biotin, and molybdenum cofactor synthesis, but they instead generate l-Ala and elemental sulfur (Van Hoewyk et al., 2008).

Emerging data over the recent years suggest that H2S may be a signaling molecule equally important to plants as NO and H2O2. H2S has been found to mediate increases in tolerance and protection against certain plant stresses. For example, sulfide alleviates the inhibitory effects of copper and aluminum stress on wheat (Triticum aestivum) germination and is associated with antioxidative defense (Zhang et al., 2008, 2010). Similarly, the inhibitory effect of boron on cucumber (Cucumis sativus) root elongation is substantially reduced by H2S treatment, which upregulates the cell wall–associated proteins and expansins (Wang et al., 2010). Moreover, sodium hydrosulfide pretreatment improves heat tolerance in tobacco (Nicotiana tabacum) suspension cultured cells, requiring the involvement of calcium and calmodulin (Li et al., 2012). It has also been reported that sulfur fertilization increases plant resistance against fungal pathogens, and it has been proposed that H2S is involved in the mechanisms of the sulfur-induced resistance, or sulfur-enhanced defense, phenomenon (Rausch and Wachter, 2005). A field experiment demonstrated that Brassica napus is able to react to fungal infection and releases H2S as a result of increased l-Cys desulfhydrase activity (Bloem et al., 2004). We recently demonstrated that cytosolic Cys plays a role in the establishment and signaling of the plant response to pathogens. This function could be due to Cys itself or to its function as generator of a particular sulfur compound (Álvarez et al., 2012). Hydrogen sulfide has been recently discovered as a component of the abscisic acid signaling network in guard cells (García-Mata and Lamattina, 2010; Lisjak et al., 2010). It has also been shown to modulate photosynthesis in spinach (Spinacia oleracea) seedlings by regulating the expression of genes involved in photosynthesis and thiol redox modification (Chen et al., 2011).

In this work, we observed senescence-associated vacuoles and an induced autophagy phenotype in des1 mutants. Additionally, we found that these phenotypes could be rescued by the exogenous application of H2S. An in-depth study of the effects of exogenous H2S revealed a specific role of sulfide as a general repressor of autophagy and a transcriptional modulator in Arabidopsis.

**RESULTS**

*Senescence-Associated Vacuoles and Induced Autophagy in des1 Mutants*

We previously demonstrated that a mutation in DES1 causes premature leaf senescence, as evidenced by increased transcript levels of senescence-associated genes (Álvarez et al., 2010). To further dissect this particular phenotype, we aimed to determine how senescence was induced at the cellular level in this mutant. Plants contain different types of vacuoles, and senescence-associated vacuoles (SAVs) are formed de novo during leaf senescence. SAVs are small in size and exhibit a lower pH than the central vacuole (Zouhar and Rojo, 2009). des1-1 mutant plants and their respective wild-type Columbia-0 (Col-0), were grown for 4 weeks side by side under long-day photoperiod and nutrient-sufficient conditions. The identification of vacuolar compartments associated with leaf senescence was performed in mesophyll protoplasts using Lysotracker Red, a fluorescent marker for acidic organelles (Otegui et al., 2005). After incubating the protoplasts prepared from des1-1 and wild-type leaves, small fluorescent structures were clearly visible in the cytoplasmic periphery around the plastids in des1-1 protoplasts but not in wild-type protoplasts (Figure 1A). Further evidence indicating that this fluorescence signal was due to the mutation in DES1 was the presence of these structures, albeit at lower abundance, in a second mutant allele, des1-2, and their absence in Nossen-0 (No-0) plants, the respective wild-type ecotype (Figure 1A).

Because cellular components are degraded and the released nutrients are mobilized for reuse during leaf senescence (Lim et al., 2007), we evaluated whether the SAVs observed in the des1 mutants were related to an autophagic mechanism. Proteins involved in autophagy (ATG proteins) have been used to monitor autophagic activity in plants, such as ATG8 accumulation and lipidation. The ATG8 protein is covalently conjugated to phosphatidylethanolamine (PE), and its lipidation is required for the formation of the autophagosomes, which is the most remarkable feature of autophagy (Nakatogawa et al., 2009). We used polyclonal antibodies raised against the recombinant ATG8 protein from Chlamydomonas reinhardtii, which has been shown to be functionally conserved and therefore useful as a molecular autophagy marker in Arabidopsis (Pérez-Pérez et al., 2010). Four-week-old des1 mutant plants and their respective wild-type ecotypes were grown as described above, leaf tissues from these plants were homogenized, and total protein samples were subjected to immunoblot analysis (Figure 1B). The antibodies detected two groups of ATG8 proteins, with a protein-banding pattern similar to those previously observed in Arabidopsis (Phillips et al., 2008; Chung et al., 2010). The slower-mobility group corresponds to unmodified protein forms, and the group with faster mobility includes ATG8 proteins conjugated with phosphatidylethanolamine. The SDS-PAGE profiles of the ATG8 proteins revealed increased accumulation in both des1 mutant alleles relative to the wild-type plants, predominantly for the modified ATG8 forms (Figure 1B). Collectively, these results suggest that deficient DES1 protein function promotes accumulation of SAVs and autophagy activation.

*Sulfide Rescues the SAV and Autophagy Phenotypes of the des1 Mutants*

DES1 catalyzes the enzymatic desulfuration of l-Cys to sulfide, and disruption of DES1 provokes a 20 to 25% increase in the amount of Cys (Álvarez et al., 2010), but it may also reduce the capacity of the cytosol to release H2S. To assess the latter, we used an H2S-selective electrode to measure endogenous H2S concentrations in leaf extracts from des1 and wild-type plants grown in soil for 4 weeks under a long-day photoperiod (Figure 2). Both des1 alleles showed a 30% reduction in endogenous sulfide compared with their respective wild-type plants, although the endogenous H2S concentration in leaves is dependent on the ecotype in Arabidopsis, with a 45 to 49% higher sulfide content in No-0 than Col-0. When plants grown under physiological conditions were treated exogenously with 200 μM Na2S for 10 d, we observed an increase in the endogenous H2S content that was limited to ∼13 pmol/mg fresh weight (FW) in Col-0 and des1-1 plants and ∼15 pmol/mg FW in No-0 and des1-2 plants (Figure 2). No phenotypic damage was observed during the period of the treatment, whose length is similar to conditions used in plants by other groups (Chen et al., 2011; Zhang et al., 2011; Dawood et al., 2012). Therefore, the quantified sulfide values do not exceed the sulfide toxicity thresholds that the plant can tolerate without compromising viability.

To determine if exogenously applied sulfide could completely or partially rescue the phenotypic characteristics observed in the des1 mutants, 20-d-old plants grown with sufficient sulfur nutrition were treated for 10 d with 200 μM Na2S and compared with untreated plants grown adjacent to them. The sulfide treatment clearly eliminated the phenotypic differences of the des1-1 mutant. After mesophyll protoplasts were prepared from the leaves of sulfide-treated plants and analyzed for the presence of SAVs, yellow fluorescent structures corresponding to SAVs stained with Lysotracker Red were not detectable in any of the high number of des1-1 protoplasts analyzed (Figure 3A). We also observed a reduced accumulation of lipidated ATG8 forms in the Na2S-treated des1-1 plants, suggesting that exogenous sulfide addition can inhibit the autophagy process (Figure 3B).

Studies assessing the role of hydrogen sulfide in mammalian systems typically involve the administration of exogenous H2S generated in vitro from both Na2S and NaHS at micromolar concentrations (Szabó, 2007). Because the NaHS compound is more widely used as the hydrogen sulfide donor, we performed experiments similar to those described above on 20-d-old plants treated with different concentrations of NaHS. We observed that exogenous addition of NaHS rescued the autophagy activation phenotype of the des1-1 mutant. Immunoblot analysis of ATG8 showed that even with a low NaHS concentration of 50 μM, the significant accumulation of modified ATG8 was lost; the protein banding patterns were similar at all NaHS concentrations (Figure 4).

To confirm that the observed phenotype of the des1-1 mutant plants was indeed due to the disruption of the DES1 gene and a reduction of cytosolic endogenous sulfide, complementation analysis was performed using the full-length DES1 cDNA fragment. We first measured and compared the endogenous H2S concentrations in leaf extracts of the complemented des1-1:P35S-DES1 line, the wild type, and the des1-1 mutant. The wild-type and complemented line contained similar levels of endogenous sulfide, 10.5 and 10.1 pmol/mg FW, respectively, but the des1-1 mutant displayed a reduced level of 6.8 pmol/mg FW. Additionally, the des1-1:P35S-DES1 line showed the same pattern of ATG8 accumulation observed in the wild type. The ATG8-phosphatidylethanolamine adducts observed in the des1-1 mutant were strongly reduced, especially considering the higher protein loading in the lanes corresponding to the wild type and complemented line (Figure 5). Therefore, complementation of des1-1 with the P35S-DES1 construct resulted in wild-type levels of endogenous sulfide and also reversed the autophagy-induced phenotype.

Further confirmation that the absence of functional DES1 in the cytosol specifically provoked the accumulation of SAVs and the induction of autophagy was the inability to detect SAVs in oas-a1.1 mesophyll protoplasts and the observation of the same patterns of ATG8 lipidation in wild-type and oas-a1.1 mutant plants (see Supplemental Figure 1 online). The oas-a1.1 mutant previously described is deficient in the most abundant OASTL isoform located in the cytosol, which dominantly contributes to the Cys biosynthesis in this compartment (López-Martín et al., 2008a). The oas-a1 mutants showed a phenotype opposite to the des1 mutants, with a significant reduction in the amount of Cys and opposite responses under pathogen attack (Álvarez et al., 2012).

In plants, autophagy is recognized as a key process in nutrient remobilization when the environmental nutrient supply is limited. Several studies have reported autophagy induction by carbon and nitrogen starvation, which is observed in many cases as a substantial increase in ATG8 protein levels (Hanaoka et al., 2002; Xiong et al., 2005; Chung et al., 2009). As indicated above, increased ATG8 protein levels were observed in des1 mutants when the plants were grown with a sufficient C, N, and S nutrient supply; thus, the effect of sulfide treatment does not appear to depend on the nutrient supply of the plant. To ensure that sulfur depletion was not a contributing factor in our plant system, we analyzed the transcript levels of genes in the sulfate assimilation pathway that are regulated by sulfur availability. Col-0 wild-type plants were grown for 20 d under the same conditions used for the previous experiments and irrigated with water or 200 μM Na2S for the next 10 d. The expression of sulfate transporter genes and APS reductases was analyzed. The transcript levels of the four sulfate transporter genes and the three APS reductases analyzed by quantitative RT-PCR (qRT-PCR) were similar for both sulfur nutrition conditions (see Supplemental Figure 2A online). These results suggest that inhibition of autophagy mediated by sulfide (as measured by ATG8 protein accumulation and modification) is unrelated to sulfur limitation.

To further reinforce our conclusion that the autophagy phenotype of the des1 mutants is completely unrelated with nutrient limitation, we analyzed the transcript levels of genes in the nitrate assimilation pathway. No significant differences were observed in the transcript levels of both nitrate transporter genes, NTR1.1 and NTR2.1, or in the expression level of NIA1 gene, encoding the minor nitrate reductase. However, we did observe in the des1-1 mutant a significant but slight induction of the NIA2 gene, encoding the major nitrate reductase (see Supplemental Figure 3 online), whose mRNA level showed to rise in response to nitrate treatment (Campbell, 1999; Wang et al., 2003).

*Sulfide Rescues the Autophagy Activation Resulting from Dark-Induced Carbon Starvation in Arabidopsis Plants*

Previous studies have shown that ATG8 mRNA accumulates when detached leaves or whole plants are placed in extended darkness to deplete the available sugars (Sláviková et al., 2005; Thompson et al., 2005; Xiong et al., 2005). To test if the effects of sulfide as an autophagy inhibitor were observable under conditions unrelated to sulfur nutrition, wild-type and des1 mutant plants grown in soil were placed in darkness to induce carbon starvation and then allowed to recover. In the absence of sulfide, enhanced chlorosis of the cotyledons and leaves was evident after the dark treatment in both the wild-type and des1 mutant plants. By contrast, sulfide addition during the dark treatment and the recovery period apparently reduced the extent of chlorosis in all plant lines, resulting in a healthier phenotype (see Supplemental Figure 4A online). Consistent with the data previously described, ATG8 protein levels, primarily the conjugated ATG8 form, increased in Col-0 wild-type plants under dark-induced carbon starvation compared with plants grown under normal physiological conditions; in the des1-1 background, a further increase in ATG8 protein accumulation was not observed (Figure 6A). Remarkably, the addition of sulfide not only reduced the levels of ATG8 proteins in the des1-1 plants grown under physiological conditions but also reduced ATG8 protein accumulation in both Col-0 and des1-1 plants under carbon starvation (Figure 6A). Nearly identical results were obtained in wild-type No-0 and des1-2 mutant plants. Sulfide also reduced the accumulation of ATG8 proteins in carbon-starved No-0 and des1-2 plants and physiologically grown des1-2 plants to the levels observed in wild-type No-0 grown under physiological conditions (Figure 6B). Further analysis of the transcript levels of four sulfate transporter genes and three APS reductase genes in Col-0 wild-type plants subjected to carbon starvation in the absence and presence of sulfide revealed no significant differences for the two sulfur conditions (see Supplemental Figure 2B online). This finding further supported that the effect of sulfide was not related to sulfur limitation.

Since DES1 catalyzes the desulfuration of l-Cys to sulfide plus ammonia, we performed the dark-induced carbon starvation experiments in the presence of ammonium to confirm the specific role of sulfide as a repressor of autophagy. Unlike sulfide, ammonium was unable to reduce chlorosis in any plant lines (see Supplemental Figure 4B online) and was unable to reduce the enhanced accumulation of ATG8 proteins in both Col-0 and des1-1 mutant plants under carbon starvation (Figure 7).

*The DES1 Mutation Significantly Alters the Transcriptional Profile at the Late Growth Stage*

Using the Affymetrix ATH1 GeneChip, we performed a comparative transcriptomic analysis on leaves of des1-1 and Col-0 plants. Total RNA was extracted from the rosette leaves of plants grown for 20 d in soil under identical long-day conditions, with three biological replicates for each genotype. These samples were used to prepare complementary RNA, which was then hybridized to the chips (Gene Expression Omnibus repository GSE19244).

The normalized data from the replicates revealed differential expression of only 16 genes in the des1-1 mutant, with all of them upregulated and none downregulated by more than twofold (see Supplemental Table 1 online). The 16 induced genes were classified into functional groups using MapMan categorization, and the most abundant group corresponded to proteins that respond to abiotic and biotic stimuli. This group included the PLANT DEFENSIN PROTEIN 1.2B (PDF1.2B) and PDF1.2A genes and CATION EXCHANGER3 (CAX3), a gene encoding the vacuolar H+/Ca2+ antiporter. Two genes closely associated with senescence in rosette leaves, SENESCENCE-ASSOCIATED GENE21 (SAG21; At4g02380) and the NAC transcription factor NAP (At1g69490), were also induced in des1-1 at this growth stage.

In another experiment, total RNA was extracted after 30 d of growth under identical conditions, and the des1-1 transcriptional profile in rosette leaves changed dramatically at this later growth stage (Gene Expression Omnibus repository GSE32566). The normalized data from the replicates showed differential expression of 1614 genes in the des1-1 mutant, with 701 genes downregulated and 913 genes upregulated by more than twofold. These differences between the transcriptional profiles of young and old des1-1 plants were clearly visible when the MA plots [differential expression of all genes as logRatio (M) plotted against their logSignal (A)] were compared (Figures 8A and 8B).

The 1614 genes with altered transcript levels were classified into 23 functional groups using MapMan categorization (see Supplemental Figure 5 online). The most highly induced genes were a Cys-type endopeptidase with unknown function (At2g27420) (71-fold induction), SAG29 (60-fold induction), which integrates environmental stress responses into the process of senescence, and LATE ELONGATED HYPOCOTYL (LHY) (50-fold induction). LHY encodes a MYB transcription factor involved in the circadian rhythm along with the MYB transcription factor encoded by CCA1, which was also induced at this stage (10-fold induction). The genes upregulated at the earlier growth stage were induced at the later growth stage with even higher levels of induction; for example, the vacuolar H+/Ca2+ antiporter gene CAX3 exhibited a 12-fold induction at the later growth stage.

Excluding genes with no functional assignments, the category “proteins” was the most important functional group and included 103 upregulated and 119 downregulated genes. Of the genes within this functional group, 41 encode proteins associated with ubiquitin- and autophagy-dependent degradation (see Supplemental Figure 6 and Supplemental Table 2 online). The autophagic recycling of intracellular constituents requires the ubiquitin-like ATG8 and ATG12 proteins (Chung et al., 2010). The genes encoding the ATG8B and ATG12A isoforms were induced in des1-1 at the later growth stage, corresponding with the induced autophagy phenotype observed in the mutant at this stage of growth. Furthermore, most of the deregulated genes in this functional group are related to ubiquitin-dependent degradation. Genes encoding proteins of the ubiquitin-proteasome system deregulated in des1-1 include three genes associated with the ubiquitin-conjugating E2 enzyme, 24 RING domain E3 ubiquitin ligase genes, and components of the SCF ubiquitin ligase complex. Among the upregulated RING domain E3 ligase genes, we identified the gene encoding the XERICO protein, which promotes abscisic acid accumulation and drought tolerance and has been identified as a DELLA target (Davière et al., 2008). The SCF ubiquitin ligase complex genes include SKP1 (At5g57900), the protein product of which functions as an adaptor to bind F-box proteins in the SCF complex, and 11 F-box genes, including AFR, which mediates the turnover of a repressor of phyA signaling (see Supplemental Table 2 online).

*Sulfide Reverses the Transcriptional Profile Changes of the des1-1 Mutant*

The effects of sulfide observed in the des1 mutants, namely, the reversal of the early senescence phenotype evidenced by the presence of SAVs and ATG8 accumulation and modification, were also observable at the transcriptional level. Total RNA was extracted from rosette leaves of des1-1 and wild-type plants grown for 20 d and treated with sodium sulfide for 10 additional days, with three biological replicates for each genotype (Gene Expression Omnibus repository GSE32566).

A comparison of the transcriptional profiles of des1-1+Na2S and Col-0+Na2S revealed that exogenous sulfide reversed differences at the transcript level between the mutant and the wild-type lines, analogous to the reversion of autophagy observed in previous experiments (Figures 8B and 8C). The transcriptional profile of the treated plants was similar to the profile at the earlier growth stage, with only six genes upregulated and nine genes downregulated by more than twofold (see Supplemental Table 3 online). The induced genes included PDF1.2B and PDF1.2A, which were also induced in untreated plants at the earlier growth stage (Figure 8A).

We performed real-time RT-PCR on a subset of genes to validate the expression data of the microarray analysis performed in plants at the late growth stage. Genes with altered expression were selected, including those with the largest fold changes in the des1-1 transcriptional profile of rosette leaves from plants grown for 30 d. The qRT-PCR analysis was performed in both mutant plants, des1-1 and des1-2, and compared with the wild-type Col-0 and No-0, respectively. Overall, there was qualitative agreement between the qRT-PCR results and the microarray analysis. The des1-1 mutant showed the same changes in gene expression; the des1-2 mutant also showed the same pattern of regulation, but with some quantitative differences (see Supplemental Figure 7 online). We also performed qRT-PCR analysis in plants grown for 20 d and treated with sodium sulfide for 10 additional days to validate the results of the transcriptomic analysis. In both mutants, we observed a reversal of the transcriptional changes of the selected genes to the expression levels observed in the wild type. Therefore, the microarray data and the results of the qRT-PCR analysis in the des1-1 and des1-2 mutants were strongly correlated (see Supplemental Figure 7 online).

The effect of the sulfide treatment on the transcriptional regulation of genes in both mutants was reinforced by the qRT-PCR analysis of the senescence-associated genes. In untreated plants, SAG21 showed low levels of transcript abundance, with higher levels in des1-2 than in des1-1, while SAG12, NAP, and PR1 had high transcript levels, and these levels were highest in des1-1. Sulfide strongly reduced most of the transcript level changes in both mutants, supporting the role of sulfide as a transcriptional regulator in the des1 mutants. SAG12, NAP, and PR1 transcripts levels were significantly reduced by sulfide treatment in both mutant plants, and SAG21 transcript level decreased in des1-2 and des1-1, but only slightly in the latter (Figure 9). Furthermore, the observed transcript level accumulation of the SA-responsive defense marker PR1 in both mutants in the absence of sulfide correlated with an increase of SA. We measured 1.6- and 1.9-fold increases in SA levels in the des1-1 and des1-2 mutants, respectively, compared with their corresponding wild-type ecotypes (see Supplemental Figure 8 online).

**DISCUSSION**

Although much attention has been given to the OASTL gene family of Arabidopsis in recent years (Heeg et al., 2008; López-Martín et al., 2008a; Watanabe et al., 2008a; Álvarez et al., 2010, 2011; Bermúdez et al., 2010; García et al., 2010; Wirtz et al., 2010), little importance has been assigned to the minor OASTL-like proteins with different enzyme activities of Cys biosynthesis, which can strongly affect plant metabolism when their function is disrupted (Gotor et al., 2010). This is the case for DES1, which is the only identified l-Cys desulfhydrase located in the cytosol and is involved in the degradation of Cys and the concomitant generation of H2S in this cellular compartment. The function of DES1 is evidenced by the fact that the T-DNA insertion mutants des1-1 and des1-2 exhibit 20 and 25% increases, respectively, in their total Cys content relative to their respective wild types (Álvarez et al., 2010). Accordingly, the leaf endogenous H2S concentrations in the null mutants are 30% less than the quantified amount in the respective wild types (this work). Assuming the volume occupied by the cytosol, which was determined to be 6.7% in barley mesophyll cells (Winter et al., 1993), the level of reduction of sulfide in the cytosol should be highly significant.

The T-DNA insertion mutants deficient in DES1 show a distinctive phenotype, whose detailed characterization has been informative about the functions of this protein related to Cys metabolism (Álvarez et al., 2010, 2012) and the role of H2S generated from Cys in the cytosol as a signaling molecule (this work). Recent work has shown that a mutation in DES1 leads to premature leaf senescence, as evidenced by the increased expression of senescence-associated genes (Álvarez et al., 2010). In this study, we provide new experimental evidence at the cellular and transcriptional levels assessing the senescence-induced phenotypes of the des1-1 and des1-2 mutants. During leaf senescence, the SAVs are formed de novo; they are smaller in size than the central vacuole, and they can be detected using specific fluorescent markers due to their acidic pH. We detected vacuoles with the same characteristics as SAVs in mesophyll protoplasts from des1 mutants, which exhibit an acidic pH, small size, and peripheral localization in the cytoplasm of cells that contain chloroplasts (Otegui et al., 2005). Our transcriptional data correlate strongly with other feature of SAVs, including their intense Cys-protease activity, and that SAG12, a papain-like Cys-protease upregulated in the des1-1 mutant, localizes to the SAVs, although other proteases besides SAG12 are targeted to this compartment (Otegui et al., 2005). The transcriptional profile of des1-1 at a later growth stage revealed that one of the most highly induced genes encodes a Cys-type endopeptidase of the family C1A located in the endomembrane system.

During leaf senescence, cellular components are recycled for redistribution from senescent leaves to younger leaves and reproductive organs. It has been shown that the ubiquitin-26S proteasome pathway mediates senescence-associated protein degradation (Yoshida, 2003; Lin and Wu, 2004), and our transcriptomic data provide further evidence that mutations in the DES1 gene promote premature senescence. Categorization of the genes with altered expression levels in the des1-1 mutant at a later growth stage indicated that the most important functional group included a high proportion of genes encoding proteins involved in the ubiquitin-dependent degradation pathway. In addition, the senescence process is also influenced by various environmental and internal factors, with the latter including phytohormones (Lim et al., 2007). SA, the hormone typically involved in the plant response to pathogens, has been implicated in leaf senescence. Higher SA levels have been reported in senescing Arabidopsis leaves, and this observation correlates with the upregulation of several SAG genes, including PR1 or SAG12 (Morris et al., 2000). Once again, our experimental data support the involvement of the SA-signaling pathway in the senescence-associated phenotype of des1 mutants because higher SA levels in both mutants were observed in addition to the accumulation of the SA-responsive defense markers PR1 and SAG12.

Links between leaf senescence and autophagy have been established, and the vast majority of homologs of the yeast ATG genes have been identified primarily in Arabidopsis and other plants (Doelling et al., 2002; Thompson et al., 2005; Yoshimoto et al., 2009). Most of the essential components are conserved, suggesting that the molecular basis of the core autophagy machinery is essentially the same in plants and yeast (Thompson and Vierstra, 2005; Bassham, 2007; Yoshimoto et al., 2010). Arabidopsis contains nine highly conserved ATG8 proteins that are associated with autophagosomes in a comparable manner (Yoshimoto et al., 2004; Sláviková et al., 2005; Chung et al., 2010). Making use of antibodies raised against recombinant ATG8 from C. reinhardtii, which have a high affinity for Arabidopsis ATG8 proteins (Perez-Perez and Crespo, 2010; Pérez-Pérez et al., 2010), we demonstrated that DES1 deficiency induces the ATG8 lipidation typically associated with the autophagy process (Yoshimoto et al., 2004; Thompson et al., 2005; Chung et al., 2010). In two ecotypic backgrounds, Col-0 and No-0, we observed that a mutation in the DES1 gene strongly promotes ATG8 protein accumulation, particularly in the lipidated form. In addition, our transcriptomic data confirm an induction of autophagy in the des1-1 mutant, in which we observed that the ATG8B and ATG12A genes were upregulated by more than twofold and that other members of the ATG8 gene family were also upregulated, including ATG8A (1.97-fold induction) and ATG8G (1.82-fold induction) (GSE32566). The abundance of ATG8 transcript levels has been shown to increase in both wild-type plants and autophagy mutants in response to Suc starvation of suspension-cultured cells (Rose et al., 2006), darkness-induced carbon starvation of intact plants (Thompson et al., 2005; Phillips et al., 2008) and detached leaves (Doelling et al., 2002), and nitrogen starvation in hydroponic media (Yoshimoto et al., 2004). Furthermore, the two ATG12 genes of Arabidopsis show distinct expression patterns: ATG12B transcripts are more abundant during early development, but ATG12A expression is higher at later growth stages and is greatly induced during leaf senescence (Chung et al., 2010).

Mutation of DES1 disrupts H2S generation in the Arabidopsis cytosol. Restoring the capacity of H2S generation, through exogenous sources (Na2S or NaHS) or by genetic complementation, eliminates the phenotypic differences of the des1 mutants from wild-type plants. Exogenous sulfide reverses the defects of des1 mutants not only at the cellular (undetectable SAVs) and protein levels (reduction of ATG8 protein accumulation and lipidation) but also at the transcriptional level. When autophagy is induced by carbon starvation, sulfide is able to revert the ATG8 protein accumulation even in wild-type plants, suggesting a general effect of sulfide on autophagy. Mutation of DES1 also disrupts the production of ammonium from Cys in the cytosol; however, exogenous ammonium did not have the same effect on autophagy as exogenous sulfide. Therefore, sulfide in particular was found to negatively regulate autophagy, and this regulation was unrelated to nutrient limitation stress. Evidence indicates that autophagy is a major mechanism for nutrient mobilization under starvation conditions in plants, and deficits of both carbon and nitrogen have been shown to induce autophagy (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005; Rose et al., 2006; Phillips et al., 2008; Chung et al., 2009). In light of the possibility that the observed effects of exogenous sulfide could be due to a compensation for nutrient starvation, we confirmed that our plant system did not exhibit sulfur or nitrogen limitation conditions. The control of sulfur assimilation occurs primarily at the steps of sulfate uptake and APS reduction, where the transcript levels of sulfate transporters and APS reductases are strictly regulated by sulfate availability and are induced under sulfur limitation conditions (Takahashi et al., 2011). We did not observe significant differences in the transcript levels of four sulfate transporters and three APS reductase genes in wild-type plants in the absence and presence of exogenous sulfide. Moreover, we did not detect any significant differences in the transcript levels of genes involved in the nitrate assimilation pathway in the des1-1 mutant plants compared with wild-type plants at the late growth stage.

Our results suggest that Cys-generated sulfide in the cytosol acts as a negative regulator of autophagy and a modulator of the transcriptional profile of Arabidopsis and that this effect is independent of the sulfur nutrient status of the plant. Subcellular sulfide concentrations have been determined in plastids (125 μM) and in the cytosol (55 μM) (Krueger et al., 2009). In plants, sulfate reduction takes place in plastids (Takahashi et al., 2011), which is consistent with the large amount of sulfide in plastids. The presence of sulfide in compartments other than plastids requires for it to be transported across membranes to those compartments. Although it has been proposed that H2S reaches the cytosol via diffusion through the chloroplast envelope membrane, the chloroplast stroma reaches a pH of 8.5 under illumination (Heldt et al., 1973; Wu and Berkowitz, 1992), conditions at which 95% of sulfide would be present in the charged HS− form. Thus, sulfide transport across the chloroplast envelope membrane may be limited (Łowicka and Beltowski, 2007; Kabil and Banerjee, 2010), which is supported by the identification and characterization of a new bacterial hydrosulfide ion channel (Czyzewski and Wang, 2012). Therefore, the sulfide in the cytosolic compartment should be metabolically generated to act as a signaling molecule.

Hydrogen sulfide is already recognized as an important signaling molecule in mammalian systems (Łowicka and Beltowski, 2007; Szabó, 2007; Gadalla and Snyder, 2010), and emerging data suggest the same for plants. It has been reported to be involved in the protection against copper, aluminum, and boron stress (Zhang et al., 2008, 2010; Wang et al., 2010) and in the regulation of photosynthesis (Chen et al., 2011). It has also been identified as a component of the ABA signaling pathway in guard cells (García-Mata and Lamattina, 2010; Lisjak et al., 2010). Our results support these findings and highlight the role of sulfide as an important regulator of autophagy. In animal systems, the mechanisms of H2S action and its molecular targets are poorly understood. H2S appears to signal predominantly through S-sulfhydrating Cys residues in its target proteins, which is analogous to S-nitrosylation by NO. In order for this posttranslation modification to occur, the Cys residue must exit in an oxidized state (e.g., as sulfenic acid or as a disulfide) and then be attacked by the hydrosulfide anion to yield a persulfide product. Whereas S-nitrosylation typically inhibits enzymes, S-sulfhydration activates them because the latter merely changes an –SH to an –SSH, enhancing the chemical reactivity of enzymes and possibly improving their access to their respective targets (Gadalla and Snyder, 2010; Kabil and Banerjee, 2010).

**METHODS**

*Plant Material, Growth Conditions, and Treatments*

Arabidopsis thaliana wild-type ecotypes Col-0 and No-0 and the SALK\_103855 (des1-1), RIKEN RATM13-27151\_G (des1-2), and SALK\_072213 (oas-a1.1) lines were used in this work (Alonso et al., 2003; Ito et al., 2005; Álvarez et al., 2010).

To generate the des1-1 complementation line, a 972-bp cDNA fragment containing the full-length coding sequence of DES1 was obtained by RT-PCR amplification using the proofreading Platinum Pfx DNA polymerase (Invitrogen) and the primers DES1F and DES1R (see Supplemental Table 4 online). The fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) and transferred into the pMDC32 vector (Curtis and Grossniklaus, 2003) using the Gateway system (Invitrogen) according to the manufacturer’s instructions. The final construct was generated by transformation into Agrobacterium tumefaciens and then introduced into des1-1 null plants using the floral-dip method (Clough and Bent, 1998).

Plants were grown in soil with a photoperiod of 16 h of white light (120 µE m−2 s−2) at 20°C and 8 h of dark at 18°C. Twenty-day-old plants were irrigated for 10 additional days with 200 µM Na2S, 50 to 200 µM NaHS, or 200 µM NH4Cl in water. The solutions were changed every 2 d. Dark-induced carbon starvation was performed on 20-d-old plants by placing them in darkness for 3 d and allowing them to recover for an additional 5 d (Thompson et al., 2005).

*Preparation of Leaf Protoplasts*

Leaves were collected from 4-week-old plants, and the Tape-Arabidopsis Sandwich experimental protocol was performed (Wu et al., 2009). The upper epidermal surface was stabilized with a strip of labeling tape (Shamrock), while the lower epidermal surface was affixed to a strip of Magic tape 3M (Scotch). The Magic tape was carefully pulled away from the labeling tape to peel away the lower epidermal surface cell layer. The peeled leaves (seven to 10 leaves) adhering to the labeling tape were transferred to a Petri dish containing 10 mL of enzyme solution (1% cellulose R10 [Serva], 0.25% macerozyme R10 [Serva], 0.4 M mannitol, 10 mM CaCl2, 20 mM KCl, 0.1% BSA, and 20 mM MES, pH 5.7). The leaves were gently shaken (40 rpm on a platform shaker) with light for 20 to 60 min until the protoplasts were released into the solution. The protoplasts were centrifuged at 100g for 3 min, washed twice with 25 mL of prechilled modified W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM Glc, and 2 mM MES, pH 5.7), and incubated on ice for 30 min. The resulting protoplast preparations were used for further analysis.

*Detection of SAVs*

Isolated protoplasts were incubated with 5 µM Lysotracker Red DND-99 (Molecular Probes) for 10 min at ambient temperature. After washing off any excess dye, samples were mounted onto a slide, with a spacer between the slide and cover slip, and observed using a TCS SP2 spectral confocal microscope (Leica Microsystems). Lysotracker Red was excited with a 543-nm helium-neon laser line, and emitted light was detected after spectral separation in the 560- to 605-nm range (pseudocolored in yellow).

*Immunoblot Analysis*

Plant leaf material (200 mg) was ground in liquid nitrogen with 400 μL of extraction buffer (100 mM Tris-HCl, pH 7.5, 400 mM Suc, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg mL−1 pepstatin A, and 4% [v/v] protease inhibitor cocktail [Roche]) using a MM400 mixer mill (Retsch) and centrifuged at 500g for 10 min to obtain the supernatant fraction as described previously (Yoshimoto et al., 2004). The total amount of protein in the resulting supernatant was determined using a method previously described (Bradford, 1976). For immunoblot analyses, 60 µg of leaf protein extracts was electrophoresed on 15% acrylamide gels before transfer to polyvinylidene fluoride membranes (Bio-Rad) according to the manufacturer’s instructions. Anti-Cr-ATG8 (Pérez-Pérez et al., 2010), and secondary antibodies were diluted 1:2000 and 1:10,000, respectively, in PBS containing 0.1% Tween 20 (Sigma-Aldrich) and 5% milk powder. The ECL-Advance immunoblotting detection system (GE Healthcare) was used to detect the proteins with horseradish peroxidase–conjugated anti-rabbit secondary antibodies.

*Real-Time RT-PCR*

Total RNA was extracted from Arabidopsis leaves using the RNeasy plant mini kit (Qiagen) and reverse transcribed using an oligo(dT) primer and the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Gene-specific primers for each gene were designed using the Vector NTI Advance 10 software (Invitrogen; see Supplemental Table 4 online), and the PCR efficiency of all primer pairs was confirmed to be close to 100%. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), and the signals were detected on an iCYCLER (Bio-Rad). The cycling profile consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A melting curve from 60 to 90°C was run following the PCR cycling. The expression levels of the genes of interest were normalized to the constitutive UBQ10 gene by subtracting the cycle threshold (CT) value of UBQ10 from the CT value of the gene (ΔCT). The fold change was calculated as 2−(ΔCT mutant − ΔCT wild type). The results shown are from three independent RNA samples.

*Microarray Hybridization and Data Analysis*

Microarray analysis of Arabidopsis ATH1 (Affymetrix) was performed as previously described (Álvarez et al., 2012). Total RNA was extracted from rosette leaves of plants grown under identical long-day conditions on soil (three biological replicates for each genotype), and these samples were used to prepare complementary RNA, which was then hybridized to the chips. Microarray analysis was performed using the affylmGUI R package (Wettenhall et al., 2006). The robust multiarray analysis algorithm was used for background correction, normalization, and summarizing expression levels (Irizarry et al., 2003). Differential expression analysis was performed using Bayes t-statistics using the linear models for microarray data (Limma), which is included in the affylmGUI package. P values were corrected for multiple testing using Benjamini and Hochberg’s method (false discovery rate) (Benjamini and Hochberg, 1995; Reiner et al., 2003). A twofold cutoff with a false discovery rate of < 0.05 and an intensity signal restriction of lgSignal > 7 were adopted to identify genes that were differentially expressed. Gene classification into functional groups was obtained from The Arabidopsis Information Resource (http://www.Arabidopsis.org) and MapMan (http://gabi.rzpd.de/projects/MapMan/).

*Measurements of Endogenous H2S*

Leaves (200 mg) were ground in liquid nitrogen to a fine powder and suspended in 150 μL of antioxidant buffer (62.5 g L−1 of sodium salicylate, 16.25 g L−1 of ascorbic acid, and 21.25 g L−1 sodium ascorbate). After vortexing for 1 min and centrifugation at 15,000g for 15 min at 4°C, H2S was measured for 20 min at 25°C in the resulting supernatant using a micro sulfide ion electrode (LIS-146AGSCM; Lazar Research Laboratories). Concentrations of H2S were determined from a calibration curve made with increasing concentrations of HNaS in antioxidant buffer. Each measurement was repeated twice, and data are from three independent biological experiments.

*Measurement of Total SA Content*

The quantification of total SA content was performed on leaves using a previously described method (Álvarez et al., 2012).

*Statistical Analysis*

The one-factor analysis of variance (ANOVA) statistical analysis of the data was performed using the program OriginPro 7.5 and the multivariate ANOVA using the program Statgraphics Centurion.

*Accession Numbers*

Sequence and microarray data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: DES1 (At5g28030); DES1 T-DNA mutants, SALK\_103855 (des1-1) and RIKEN RATM13-27151\_G (des1-2); and OAS-A1 T-DNA mutant, SALK\_072213 (oas-a1.1). The microarray Gene Expression Omnibus accession numbers are GSE19244 and GSE32566.

**Acknowledgments**

This work was funded in part by the European Regional Development Fund through the Ministerio de Ciencia e Innovación (Grants BIO2010-15201 to C.G. and BFU2009-07368 to J.L.C.) and the Junta de Andalucía (Grant BIO-273). This work was also funded by the CONSOLIDER CSD2007-00057, Spain, and through fellowship support from the Junta para la Ampliación de Estudios program (Consejo Superior de Investigaciones Científicas) awarded to C.A. We thank María Ángeles Bermúdez for the SA content determination.

AUTHOR CONTRIBUTIONS

C.A. and I.M. performed research and analyzed data. I.G., J.L.C., and L.C.R. designed the research, analyzed data, and contributed to the discussion, M.E.P.-P. contributed the antiCrATG8 antibodies and analyzed data. C.G. designed the research, analyzed data, and wrote the article.

**References**

1. Alonso J.M., et al. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653–657.

2. Álvarez C., Bermúdez M.A., Romero L.C., Gotor C., García I. (2012). Cysteine homeostasis plays an essential role in plant immunity. New Phytol. 193: 165–177.

3. Álvarez C., Calo L., Romero L.C., García I., Gotor C. (2010). An O-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. Plant Physiol. 152: 656–669.

4. Alvarez C., Lozano-Juste J., Romero L.C., García I., Gotor C., León J. (2011). Inhibition of Arabidopsis O-acetylserine(thiol)lyase A1 by tyrosine nitration. J. Biol. Chem. 286: 578–586.

5. Bassham D.C. (2007). Plant autophagy—More than a starvation response. Curr. Opin. Plant Biol. 10: 587–593.

6. Benjamini Y., Hochberg Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Statist. Soc. B 57: 289–300.

7. Bermúdez M.A., Páez-Ochoa M.A., Gotor C., Romero L.C. (2010). Arabidopsis S-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control. Plant Cell 22: 403–416.

8. Bloem E., Riemenschneider A., Volker J., Papenbrock J., Schmidt A., Salac I., Haneklaus S., Schnug E. (2004). Sulphur supply and infection with Pyrenopeziza brassicae influence L-cysteine desulphydrase activity in Brassica napus L. J. Exp. Bot. 55: 2305–2312.

9. Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

10. Campbell W.H. (1999). Nitrate reductase structure, function, and regulation: Bridging the gap between biochemistry and physiology. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 277–303.

11. Chen J., Wu F.H., Wang W.H., Zheng C.J., Lin G.H., Dong X.J., He J.X., Pei Z.M., Zheng H.L. (2011). Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in Spinacia oleracea seedlings. J. Exp. Bot. 62: 4481–4493.

12. Chung T., Phillips A.R., Vierstra R.D. (2010). ATG8 lipidation and ATG8-mediated autophagy in Arabidopsis require ATG12 expressed from the differentially controlled ATG12A AND ATG12B loci. Plant J. 62: 483–493.

13. Chung T., Suttangkakul A., Vierstra R.D. (2009). The ATG autophagic conjugation system in maize: ATG transcripts and abundance of the ATG8-lipid adduct are regulated by development and nutrient availability. Plant Physiol. 149: 220–234.

14. Clough S.J., Bent A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16: 735–743.

15. Curtis M.D., Grossniklaus U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol. 133: 462–469.

16. Czyzewski B.K., Wang D.N. (2012). Identification and characterization of a bacterial hydrosulphide ion channel. Nature 483: 494–497.

17. Davière J.M., de Lucas M., Prat S. (2008). Transcriptional factor interaction: A central step in DELLA function. Curr. Opin. Genet. Dev. 18: 295–303.

18. Dawood M., Cao F., Jahangir M.M., Zhang G., Wu F. (2012). Alleviation of aluminum toxicity by hydrogen sulfide is related to elevated ATPase, and suppressed aluminum uptake and oxidative stress in barley. J. Hazard. Mater. 209-210: 121–128.

19. Doelling J.H., Walker J.M., Friedman E.M., Thompson A.R., Vierstra R.D. (2002). The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in Arabidopsis thaliana. J. Biol. Chem. 277: 33105–33114.

20. Gadalla M.M., Snyder S.H. (2010). Hydrogen sulfide as a gasotransmitter. J. Neurochem. 113: 14–26.

21. García I., Castellano J.M., Vioque B., Solano R., Gotor C., Romero L.C. (2010). Mitochondrial beta-cyanoalanine synthase is essential for root hair formation in Arabidopsis thaliana. Plant Cell 22: 3268–3279.

22. García-Mata C., Lamattina L. (2010). Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. New Phytol. 188: 977–984.

23. Gotor C., Alvarez C., Bermúdez M.A., Moreno I., García I., Romero L.C. (2010). Low abundance does not mean less importance in cysteine metabolism. Plant Signal. Behav. 5: 1028–1030.

24. Hanaoka H., Noda T., Shirano Y., Kato T., Hayashi H., Shibata D., Tabata S., Ohsumi Y. (2002). Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene. Plant Physiol. 129: 1181–1193.

25. Heeg C., Kruse C., Jost R., Gutensohn M., Ruppert T., Wirtz M., Hell R. (2008). Analysis of the Arabidopsis O-acetylserine(thiol)lyase gene family demonstrates compartment-specific differences in the regulation of cysteine synthesis. Plant Cell 20: 168–185.

26. Heldt W.H., Werdan K., Milovancev M., Geller G. (1973). Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. Biochim. Biophys. Acta 314: 224–241.

27. Howarth J.R., Domínguez-Solís J.R., Gutiérrez-Alcalá G., Wray J.L., Romero L.C., Gotor C. (2003). The serine acetyltransferase gene family in Arabidopsis thaliana and the regulation of its expression by cadmium. Plant Mol. Biol. 51: 589–598.

28. Irizarry R.A., Hobbs B., Collin F., Beazer-Barclay Y.D., Antonellis K.J., Scherf U., Speed T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4: 249–264.

29. Ito T., Motohashi R., Kuromori T., Noutoshi Y., Seki M., Kamiya A., Mizukado S., Sakurai T., Shinozaki K. 2005). A resource of 5,814 dissociation transposon-tagged and sequence-indexed lines of Arabidopsis transposed from start loci on chromosome 5. Plant Cell Physiol. 46: 1149–1153.

30. Kabil O., Banerjee R. (2010). Redox biochemistry of hydrogen sulfide. J. Biol. Chem. 285: 21903–21907.

31. Krueger S., Niehl A., Lopez Martin M.C., Steinhauser D., Donath A., Hildebrandt T., Romero L.C., Hoefgen R., Gotor C., Hesse H. (2009). Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in Arabidopsis. Plant Cell Environ. 32: 349–367.

32. Li Z.G., Gong M., Xie H., Yang L., Li J. (2012). Hydrogen sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (Nicotiana tabacum L) suspension cultured cells and involvement of Ca(2+) and calmodulin. Plant Sci. 185-186: 185–189.

33. Lim P.O., Kim H.J., Nam H.G. (2007). Leaf senescence. Annu. Rev. Plant Biol. 58: 115–136.

34. Lin J.F., Wu S.H. (2004). Molecular events in senescing Arabidopsis leaves. Plant J. 39: 612–628.

 35. Lisjak M., Srivastava N., Teklic T., Civale L., Lewandowski K., Wilson I., Wood M.E., Whiteman M., Hancock J.T. (2010). A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. Plant Physiol. Biochem. 48: 931–935.

36. López-Martín M.C., Becana M., Romero L.C., Gotor C. (2008a). Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in Arabidopsis. Plant Physiol. 147: 562–572.

37. López-Martín M.C., Romero L.C., Gotor C. (2008b). Cytosolic cysteine in redox signaling. Plant Signal. Behav. 3: 880–881.

38. Łowicka E., Bełtowski J. (2007). Hydrogen sulfide (H2S) - The third gas of interest for pharmacologists. Pharmacol. Rep. 59: 4–24.

39. Morris K., MacKerness S.A., Page T., John C.F., Murphy A.M., Carr J.P., Buchanan-Wollaston V. (2000). Salicylic acid has a role in regulating gene expression during leaf senescence. Plant J. 23: 677–685.

40. Nakatogawa H., Suzuki K., Kamada Y., Ohsumi Y. (2009). Dynamics and diversity in autophagy mechanisms: Lessons from yeast. Nat. Rev. Mol. Cell Biol. 10: 458–467.

41. Otegui M.S., Noh Y.S., Martínez D.E., Vila Petroff M.G., Staehelin L.A., Amasino R.M., Guiamet J.J. (2005). Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean. Plant J. 41: 831–844.

42. Perez-Perez M.E., Crespo J.L. (2010). Autophagy in the model alga Chlamydomonas reinhardtii. Autophagy 6: 562–563.

43. Pérez-Pérez M.E., Florencio F.J., Crespo J.L. (2010). Inhibition of target of rapamycin signaling and stress activate autophagy in Chlamydomonas reinhardtii. Plant Physiol. 152: 1874–1888.

44. Phillips A.R., Suttangkakul A., Vierstra R.D. (2008). The ATG12-conjugating enzyme ATG10 is essential for autophagic vesicle formation in Arabidopsis thaliana. Genetics 178: 1339–1353.

45. Rausch T., Wachter A. (2005). Sulfur metabolism: A versatile platform for launching defence operations. Trends Plant Sci. 10: 503–509.

46. Reiner A., Yekutieli D., Benjamini Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics 19: 368–375.

47. Rose T.L., Bonneau L., Der C., Marty-Mazars D., Marty F. (2006). Starvation-induced expression of autophagy-related genes in Arabidopsis. Biol. Cell 98: 53–67.

48. Sláviková S., Shy G., Yao Y., Glozman R., Levanony H., Pietrokovski S., Elazar Z., Galili G. (2005). The autophagy-associated Atg8 gene family operates both under favourable growth conditions and under starvation stresses in Arabidopsis plants. J. Exp. Bot. 56: 2839–2849.

49. Szabó C. (2007). Hydrogen sulphide and its therapeutic potential. Nat. Rev. Drug Discov. 6: 917–935.

50. Takahashi H., Kopriva S., Giordano M., Saito K., Hell R. (2011). Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu. Rev. Plant Biol. 62: 157–184.

51. Thompson A.R., Doelling J.H., Suttangkakul A., Vierstra R.D. (2005). Autophagic nutrient recycling in Arabidopsis directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol. 138: 2097–2110.

52. Thompson A.R., Vierstra R.D. (2005). Autophagic recycling: Lessons from yeast help define the process in plants. Curr. Opin. Plant Biol. 8: 165–173.

53. Van Hoewyk D., Pilon M., Pilon-Smits E.A.H. (2008). The functions of NifS-like proteins in plant sulfur and selenium metabolism. Plant Sci. 174: 117–123.

54. Wang B.L., Shi L., Li Y.X., Zhang W.H. (2010). Boron toxicity is alleviated by hydrogen sulfide in cucumber (Cucumis sativus L.) seedlings. Planta 231: 1301–1309.

55. Wang R., Okamoto M., Xing X., Crawford N.M. (2003). Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol. 132: 556–567.

56. Watanabe M., Kusano M., Oikawa A., Fukushima A., Noji M., Saito K. (2008a). Physiological roles of the beta-substituted alanine synthase gene family in Arabidopsis. Plant Physiol. 146: 310–320.

57. Wettenhall J.M., Simpson K.M., Satterley K., Smyth G.K. (2006). affylmGUI: A graphical user interface for linear modeling of single channel microarray data. Bioinformatics 22: 897–899.

58. Winter H., Robinson D.G., Heldt H.W. (1993). Subcellular volumes and metabolite concentrations in barley leaves. Planta 191: 180–190.

59. Wirtz M., et al. (2010). Structure and function of the hetero-oligomeric cysteine synthase complex in plants. J. Biol. Chem. 285: 32810–32817.

60. Wirtz M., Droux M., Hell R. (2004). O-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in Arabidopsis thaliana. J. Exp. Bot. 55: 1785–1798.

61. Wu F.H., Shen S.C., Lee L.Y., Lee S.H., Chan M.T., Lin C.S. (2009). Tape-Arabidopsis Sandwich - A simpler Arabidopsis protoplast isolation method. Plant Methods 5: 16.

62. Wu W., Berkowitz G.A. (1992). Stromal pH and photosynthesis are affected by electroneutral K and H exchange through chloroplast envelope ion channels. Plant Physiol. 98: 666–672.

63. Xiong Y., Contento A.L., Bassham D.C. (2005). AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis thaliana. Plant J. 42: 535–546.

64. Yoshida S. (2003). Molecular regulation of leaf senescence. Curr. Opin. Plant Biol. 6: 79–84.

65. Yoshimoto K., Hanaoka H., Sato S., Kato T., Tabata S., Noda T., Ohsumi Y. (2004). Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell 16: 2967–2983.

66. Yoshimoto K., Jikumaru Y., Kamiya Y., Kusano M., Consonni C., Panstruga R., Ohsumi Y., Shirasu K. (2009). Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis. Plant Cell 21: 2914–2927.

67. Yoshimoto K., Takano Y., Sakai Y. (2010). Autophagy in plants and phytopathogens. FEBS Lett. 584: 1350–1358.

68. Zhang H., Hu L.Y., Hu K.D., He Y.D., Wang S.H., Luo J.P. (2008). Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. J. Integr. Plant Biol. 50: 1518–1529.

69. Zhang H., Hu S.-L., Zhang Z.-J., Hu L.-Y., Jiang C.-X., Wei Z.-J., Liu J., Wang H.-L., Jiang S.-T. (2011). Hydrogen sulfide acts as a regulator of flower senescence in plants. Postharvest Biol. Technol. 60: 251–257.

70. Zhang H., Tan Z.Q., Hu L.Y., Wang S.H., Luo J.P., Jones R.L. (2010). Hydrogen sulfide alleviates aluminum toxicity in germinating wheat seedlings. J. Integr. Plant Biol. 52: 556–567.

71. Zouhar J., Rojo E. (2009). Plant vacuoles: Where did they come from and where are they heading? Curr. Opin. Plant Biol. 12: 677–684.

**Figure captions**

**Figure 1**. Early Senescence and Induced Autophagy Phenotypes of des1 Mutant Plants.

(A) Wild-type Col-0 and No-0 ecotypes and des1 mutant plants were grown in soil for 4 weeks, and SAVs were visualized by staining of protoplasts isolated from leaves with Lysotracker Red. The yellow fluorescence due to specific staining is superimposed on the chlorophyll-specific red fluorescence. The experiment was repeated at least four times with different batches of plants, and the results were consistent across all replicates.

(B) Immunoblot analysis of ATG8 accumulation in leaves of Col-0 and No-0 wild-type and des1 mutant plants. The total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-ATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least four times with different batches of plants, and a representative image is shown.

**Figure 2**. Endogenous H2S Content in Leaf Extracts.

Twenty-day-old Col-0 and des1-1 (A) and No-0 and des1-2 (B) plants grown in soil under physiological conditions were divided into two batches. One batch was maintained at the same conditions (No addition), and the other was irrigated with 200 μM Na2S for 10 additional days (+ Exogenous Na2S). After this period, leaf extracts were prepared, and electrochemical detection of H2S was performed using an H2S-selective electrode as described in Methods. Data are from three independent experiments and two-factor ANOVA was conducted. Same letters indicate no statistical differences. P < 0.05.

**Figure 3**. Effect of Exogenous Sulfide on the SAVs and Induced Autophagy Phenotype of des1-1 Mutant Plants.

(A) Twenty-day-old Col-0 and des1-1 plants were grown in soil and irrigated with water (no addition) or with 200 μM Na2S in water for 10 d, and SAVs were visualized by Lysotracker Red staining of protoplasts isolated from leaves; this staining was superimposed on red chlorophyll fluorescence. The experiment was repeated at least four times with different batches of plants, and the results were consistent across replicates. A representative image is shown.

(B) Immunoblot analysis of ATG8 accumulation in leaves of Col-0 and des1-1 plants treated with 200 μM Na2S for 10 d and untreated plants (no Na2S addition) grown side by side. The total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-ATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least four times with different batches of plants, and a representative image is shown.

**Figure 4**. Effect of Exogenous NaHS on the Induced Autophagy Phenotype of the des1-1 Mutant.

Twenty-day-old Col-0 and des1-1 plants grown in soil were irrigated with water (no addition) or with different concentrations of NaHS in water for 10 d, and immunoblot analysis of ATG8 accumulation in leaves was performed. The total lysates were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-ATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least four times with different batches of plants, and a representative image is shown.

**Figure 5**. Genetic Complementation of the Induced Autophagy Phenotype of the des1-1 Mutant.

Immunoblot analysis of ATG8 accumulation in the leaves of 4-day-old Col-0, des1-1, and the complemented des1-1:P35S-DES1 plants. The total lysates were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-ATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least two times with different batches of plants, and a representative image is shown.

**Figure 6**. Effect of Exogenous Sulfide on the Induced Autophagy Phenotype of Arabidopsis Plants Subjected to Dark-Induced Carbon Starvation.

Twenty-day-old Col-0 and des1-1 (A) and No-0 and des1-2 (B) plants were grown in soil under physiological conditions and irrigated with water or 200 μM Na2S for 10 d before leaves were collected. Another batch of the same plant lines was subjected to carbon starvation in the absence or presence of 200 μM Na2S by placing it in darkness for 3 d and allowing it to recover for 5 d before the leaves were collected. Total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-ATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least three times with different batches of plants, and a representative image is shown.

**Figure 7**. Effect of Exogenous Ammonium on the Induced Autophagy Phenotype of Arabidopsis Plants Subjected to Dark-Induced Carbon Starvation.

Twenty-day-old Col-0 and des1-1 plants grown in soil were subjected to carbon starvation in the absence or presence of 200 μM NH4Cl by placing them in darkness for 3 d and allowing them to recover for an additional 5 d. Total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-ATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least three times with different batches of plants, and a representative image is shown.

**Figure 8.** MA Plots of Normalized Transcript Values.

Using the Affymetrix ATH1 GeneChips, we performed a comparative transcriptomic analysis on leaves of des1-1 and Col-0 plants grown under identical long-day conditions in soil for 20 d (A), 30 d (B), or 20 d with an additional 10 d of treatment with 200 μM Na2S (C). The differential expression (log ratio) of all genes that were differentially regulated in the des1-1 mutant (y axis) were plotted against their log signal (x axis). Red and green dots represent genes upregulated and downregulated, respectively, by more than twofold, with an FDR of < 0.05 and an intensity signal restriction of lgSignal > 7. MA, differential expression of all genes as logRatio (M) plotted against their logSignal (A).

**Figure 9**. Relative Expression of Senescence-Associated Genes in des1 Mutant Plants.

Real-time RT-PCR analysis of NAP (At1g69490), PR1 (At2g14610), SAG12 (AT5G45890), and SAG21 (At4g02380) was performed in leaves from des1-1 (A) and des1-2 (B) mutant plants and their respective wild types. Mutant and wild-type plants were grown under identical long-day conditions in soil for 30 d (dark-gray triangles) or for 20 d with 200 μM Na2S treatment for 10 additional days (light-gray triangles), as indicated in the figure. The transcript levels were normalized to the constitutive UBQ10 gene. Data shown are from three independent analyses and represent the transcript levels of each gene in the des1 mutant plant relative to the respective wild-type ecotype. ANOVA was performed and asterisks represent statistical differences in gene expression between the mutant and the wild type (P < 0.05).

Figure 1



Figure 2



Figure 3



Figure 4



Figure 5



Figure 6



Figure 7



Figure 8



Figure 9

